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# Production of bioemulsifier by *Bacillus subtilis*, *Alcaligenes faecalis* and *Enterobacter* species in liquid culture

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**Abstract**

Three bacterial strains isolated from waste crude oil were selected due to their capacity of growing in the presence of hydrocarbons and production of bioemulsifier. The genetic identification (PCR of the 16S rDNA gene using fD1 and rD1 primers) of these strains showed their affiliation to *Bacillus subtilis*, *Alcaligenes faecalis* and *Enterobacter* sp. These strains were able to emulsify *n*-octane, toluene, xylene, mineral oils and crude oil, look promising for bioremediation application. Finally, chemical composition, emulsifying activity and surfactant activity of the biopolymers produced by the selected strains were studies under different culture conditions. Our results showed that chemical and functional properties of the bioemulsifiers were affected by the carbon source added to the growth media.


*Keywords:* *Bacillus subtilis*; *Alcaligenes faecalis*; *Enterobacter*; Bioemulsifiers; Hydrocarbon

## 1. Introduction

Oil contamination with petroleum hydrocarbons has caused critical environmental and health effects. Various processes have been developed to remove oil from contaminated areas and there is an increased interest in promoting environmental responsibility through cleaning and reusing products that have traditionally discarded after a single use.

Generally, hydrocarbons from crude oils and refined products are biodegradable. Bioremediation of oil polluted environment is carried out by microorganisms which are generally ubiquitous in nature and with the ability of using different types of hydrocarbons as carbon and energy source. But, the low solubility in water of these compounds is one of the major limiting factors in the biodegradation process. For this reason, bacteria growing on oil polluted sites often produce emulsifiers to increase the bioavailability of hydrocarbons and to facilitate their uptake (Desai and Banat, 1997; Ron and Rosenberg, 2002).

Chemical surfactants and emulsifiers can be used to enhance the bioavailability of hydrophobic compounds, to desorb them from solid surface or to increase the apparent water solubility. However; most of these surfactants chemically synthesized are toxic to environments, not easily biodegradable and their manufacturing processes and by-products can be environmentally hazardous (Maier and Soberon-Chavez, 2000). In contrast, biosurfactants, are biodegradable, have low toxicity and have shown efficient functionality under extreme conditions, that is why these biopolymers have being more and more used in soil washing and in oil removal from contaminated areas. Certainly, environmental compatibility is nowadays becoming progressively more significant characteristic (Calvo et al., 2004; Kosaric, 1992; Mulligan et al., 2001; Ron and Rosenberg, 2002).

Biosurfactant are produced by a wide variety of microorganisms. These microorganisms produce low molecular weight polymers that efficiently reduce surface and interfacial tensions and high molecular weight molecules which

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are highly efficient emulsifiers (Banat et al., 2000). The high molecular weight biopolymers are composed of carbohydrates, proteins, lipids and other organic and inorganic compounds. These exopolymers are often produced during stationary phase bacterial growth and they exhibit considerable substrate specificity. For example, some emulsify mixture of aliphatic hydrocarbons but will not emulsify pure aliphatic, other can only emulsify pure hydrocarbons of a high molecular weight (Banat et al., 2000; Calvo et al., 2002).

Chemical composition and emulsifying activity of the biosurfactant depend not only of the producer strain but also on the culture conditions. Thus, the nature of the carbon source, the nitrogen source as well as the C:N ratio, nutritional limitations and chemical and physical parameters such as temperature, aeration and pH influence not only the amount but also the type of polymer produced. (Desai and Banat, 1997; Martínez-Checa et al., 2002; Mulligan and Gibbs, 1989).

This study was undertaken to characterize the extracellular bioemulsifiers synthesized by three bacterial strains isolated in our laboratory from waste crude oil (Calvo et al., 2002). We have studied chemical composition, emulsifying activity, surfactant activity of these exopolymers and the effect of various hydrocarbons on the functional properties of biopolymers. The main objective of this research was to point out the efficacy to enhance a bioremediation process of both bioemulsifiers and emulsifiers producing strains.

## 2. Methods

### 2.1. Microorganisms

Strains 28, 212, 214 were isolated in our laboratory from solid waste crude oil samples collected from the clean up of oil storage containers and grew in liquid media amended with polycyclic aromatic hydrocarbons according to Calvo et al. (2002).

### 2.2. Culture medium

Production of exopolysaccharide (EPS) was studied according to Toledo et al. (2006) in nutrient broth (NB) medium with the following composition (g $L^{-1}$): glucose 10, proteose peptone 5, yeast extract 5 and NaCl 5 (pH 7.2).

Influence of hydrocarbons on EPS yield production and on the properties of biopolymers was studied in the above mentioned NB medium supplemented with 1% (v/v) of the following hydrophobic substrates: *n*-octane, toluene, xylene, mineral light oil, mineral heavy oil (Sigma) and crude oil (Kirkuk) provide by Repsol YPF (Puertollano, Spain).

### 2.3. Preliminary characterization and EPS production

Production of EPS by strains 28, 212 and 214 was studied according to methods previously reported by Calvo et al. (1998). Erlenmeyer flasks of 500 ml containing 100 ml of NB medium were inoculated with 1 ml of a 24 h culture of microorganisms, grown in the same medium at 32 °C and 100 rpm. After incubation at 32 °C for 8 days, the cultures were centrifuged at 36,000*g* in a Sorval RC-5B refrigerated centrifuge at 4 °C for 60 min. Supernatants obtained were precipitate with three volumes of cold ethanol for the isolation of exopolysaccharides. The biopolymers of the precipitated were dissolved in distilled water, dialysed against distilled water during 24 h, lyophilized and then weighed.

Production of exopolymers was also studied in NB medium amended with 1% (v/v) *n*-octane, toluene, xylene, mineral light oil, mineral heavy oil (Sigma) and crude oil (Kirkuk). EPS production in hydrocarbons media were also determinate under the same conditions that above describe.

Carbohydrate and protein contents of the isolated biopolymers were determined by colorimetric assays according the methodology described by Dubois et al. (1956) and Bradford (1976).

### 2.4. Emulsification assays and surfactant activity test

The emulsifying activity of the EPSs synthesized by the selected strains grown in NB liquid media amended or unamended with various hydrocarbons was detected by a modified version of the method previously described by Cooper and Goldenberg (1987). Test tubes (105 × 15 mm) were amended with 3 ml of EPS diluted in distilled water (0.1% w/v) and 3 ml of a hydrophobic substrate (*n*-octane, toluene, xylene, mineral light oil, mineral heavy oil and crude oil). Then the tubes were shaken vigorously to homogeneity using a vortex and left to stand for 24 h. Emulsifying activity was expressed as the percentage of the total height occupied by the emulsion.

The surface tension of the produced biopolymers produced by the selected strain was measured with a Krüs K11 digital tensiometer, using a plate method (Barathi and Vasudevan, 2001). EPS lyophiles were dissolved in deionized water at concentration ranging from 0.05% to 1% (w/v) and 10 ml of each solution were place into small Petri dishes for the surface tension assays.

### 2.5. Genetic identification of strains

The strains tested in this study were identified by the analysis of the sequence of the gene encoding 16S rRNA (16S rDNA). Primers, fD1 and rD1 were used to amplify almost the full length of 16S rRNA gene from each strain (Weisburg et al., 1991). A fresh cultured colony of each strain grown on TSA medium was lysated by the addition of 20 µl of a mixture of NaOH (0.05 M)–SDS (0.25%, w/v) and then boiled for 15 min. The lysates were adjusted to 200 µl with sterile bidistilled water and centrifuged at 13,000 rpm for 5 min. in a tabletop centrifuge.